HSB Project 3

Benzene Low Dose Inhalation Induced Hematotoxicity and Genotoxicity Phenotypes and Haplotype Association Analyses

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Background and Rationale

Benzene is an important industrial chemical (> 2 billion gallons produced annually in U.S.) and it is a component of gasoline and other fuels, tobacco combustion, foods, and beverages. Benzene exposure is an established cause of human hematopoietic disease (Aksoy, 1989; Landrigan, 1987; Landrigan, 1990; Rinsky *et al.*, 1987). Also, the mechanism of benzene-induced hematotoxicity and genotoxicity remain unclear, especially with regard to the role of host (genetic and epigenetic) and environmental determinants (Lan *et al.*, 2004; Lan *et al.*, 2005; Landrigan, 1987; McHale *et al.*, 2008; Ross, 1996; Smith *et al.*, 2004). The association of benzene with non-Hodgkin lymphoma (NHL) also requires further examination through molecular epidemiological and mechanistic approaches that would be informed by the use of appropriate experimental animal models (Steinmaus *et al.*, 2008; Wong and Raabe, 2000).

Recently, significant findings have shown that: (1) low occupational levels of benzene exposure, below the current US occupational standard of 1 ppm, were toxic to hematopoietic stem cells (Lan *et al.*, 2004); (2) benzene-induced toxicity and leukemia-specific chromosome changes were observed in myeloid progenitor cells of exposed workers (McHale *et al.*, 2008); (3) low occupational levels of benzene exposure produced multiple small, but significant changes in gene expression in circulating blood cells with hematopoietic differentiation potential (McHale *et al.*, 2009); and (4) polymorphic variants in cytokine and DNA repair genes were associated with increased susceptibility to benzene hematotoxicity (Kim *et al.*, 2007; Lan *et al.*, 2009; Lan *et al.*, 2005; Shen *et al.*, 2006). Meta-analysis of the benzene toxicity literature has revealed conflicting evidence of an association between occupational benzene exposure and non-Hodgkins lymphoma (NHL) (Steinmaus *et al.*, 2008; Wong and Raabe, 2000). Furthermore, preliminary results from studies in progress investigating the differences between multiple inbred strains for absorption, distribution, metabolisms, and excretion (ADME) of oral exposure to benzene suggest significant differences between inbred strains that may affect toxicity and disease phenotypes at low exposures relevant to human occupational and environmental exposures.

Key Issues

Benzene toxicity is greatest to the rodent and human hematopoietic system, but the bulk of metabolism of benzene and its reactive intermediates occurs in the liver. At high doses and repeat exposures, stable active intermediates are produced in the liver that are distributed to tissues where damage to the hematopoietic tissue and circulating blood cells and stem cells have been observed. Benzene is metabolized to a number of toxic and genotoxic intermediate metabolites by phase 1 and phase 2 enzymes that are

eliminated in the breath, urine, and feces. Even after many years of investigation, no consensus has been reached that describe how the liver (a major source of benzene metabolites) and the skin and bone marrow (a source of minor but very toxic metabolites) interact and result in toxicity and disease upon repeated exposures.

1. Effects of route of exposure, dose and dose rate (intermittent versus continuous) on hematotoxicity and genotoxicity. In consideration of the route of exposure and dose levels, it is important to note that inhalation of benzene results in a slower rate of delivery and a greater internal dose. At equivalent oral dose (mg/kg), more benzene is expired unmetabolized after administration by the oral route (60%) than by inhalation (14%). Mice may also produce a greater variety of benzene metabolites by inhalation (Henderson *et al.*, 1989; Rappaport *et al.*, 2009; Sabourin *et al.*, 1989; Sabourin *et al.*, 1987) that might be influenced by alternate pathways of metabolism (Rappaport *et al.*, 2009).

   Selection of exposure level is critical for several reasons. High exposure levels may become saturating to metabolic pathways of detoxification. For inhalation exposure to rats and mice, this occurs at 200 ppm (6 h time-weighted average or TWA). Between 5 and 50 ppm benzene (6 h TWA), there is no significant difference between urinary metabolites (Sabourin *et al.*, 1989). Low dose (1 ppm or less), repeated exposure have not been sufficiently investigated. Available data also suggest that the ratio of hydroquinone or muconic acid to phenol ratio after inhalation exposure to less than 50 ppm (6 h TWA) is closer between mice and humans than either rats or Cynomolgus monkeys. The liver metabolizes the greatest mass of distributed benzene, but other tissues (lung, bone marrow or extramedullary hematopoietic tissues in the mouse spleen) may also metabolize benzene at low exposure levels where saturation of metabolism does not occur. Thus, high-level exposures may be metabolism differently from low dose exposures and result in differences in tissue specific metabolites and toxicity (Lan *et al.*, 2004).

2. Evidence suggests that tissue specific metabolic pathways and toxicity (liver versus bone marrow) are possible. The liver with increased mass and metabolic capacity may not be easily be saturated and is known to produce significantly more conjugated products, whereas the hematopoietic tissue may be easily saturated due to the lack of metabolic capacity (Henderson *et al.*, 1989). Evidence suggests that 2 or more pathways may exist that may determine toxicity depending upon the route of benzene metabolism and disposition (Rappaport *et al.*, 2009; Rappaport *et al.*, 2005).

3. Generation of stable, low dose exposures of 10 ppm or less of benzene in the atmosphere are difficult to reproduce over extended periods for precise inhalation exposures. This is critical because low exposures may produce the most toxic benzene metabolite intermediates. Thus, an exposure model that takes into account saturation of benzene metabolism pathways, data on exposures and high-affinity, low capacity pathways of metabolism that result in the most hematotoxic intermediates should be developed. With increased sensitive analytical equipment,

preliminary studies have indicated that a 1 ppm atmosphere of benzene may be generated for extended periods. Appropriate quality control measurements will document any excursions from target dose levels (6 h time weighted averages) and incorporated into the data analysis.

Hypothesis

Genetic variants (SNP and/or structural) between strains determine the exposure level dependent tissue specific metabolism of benzene and tissue-specific benzene toxicity.

Approach and Specific Aims

Individual differences in human or rodent ADME and toxicity to inhaled benzene have not been investigated. The use of multiple inbred strains of mice with observed differences in ADME kinetic parameters (oral exposure) and other genetically diverse mice is warranted and will allow us to determine the variable range for both hematotoxicity and genotoxicity to inhaled benzene at low levels of exposure. Using a genetic analysis of the complex toxicity phenotypes will allow identification of quantitative trait loci that can be used to identify potential candidate genes. Bioinformatic analysis of mouse candidate genes may identify human orthologs that can be further investigated and functionally validated. These results may be used to inform molecular epidemiology investigations of the benzene exposed human population. This is not possible with current *in vivo* models or cell based *in vitro* models of benzene toxicity.

Inbred laboratory mouse strains show a high degree of homology and synteny to the human and are the primary experimental genetic model for human disease. In part, this is due to the recombinant DNA methodologies that allow genetic alteration (knockouts, knockin, and conditional expression of target sequences based upon tissue specific promoters using the cre-lox system). Many human disease associated single nucleotide polymorphisms (SNPs) have led to the discovery of the exact same SNP variant and similar disease in mouse orthologs. Conversely, causally related SNPs in mice have led to the discovery of the human orthologs associated with the same disease. The limitations of gene orthology and essential genes have not been defined. Thus, the mouse is the most appropriate species for comparative genetics with well-defined methodological tools for genetics, molecular biology, statistics, and bioinformatics.

To estimate toxicity of benzene to the blood forming system in the bone marrow and circulating blood cells in each exposure group, we will phenotype up to 34 inbred strains of male mice to low levels of inhaled benzene (0, 1, 10, or 100 ppm) for 28 days and examine the effect of genetic variation on hemato- and genotoxicity. This multiple strain study population is being used to model low dose human exposures to benzene. Sufficient blood will be removed under carbon dioxide asphyxiation followed by terminal exsanguination. Anticoagulated blood and marrow will be used to quantitatively determine total erythrocyte and nucleated cell counts and a blood smear prepared for a differential (different nucleated cell types) and absolute cell counts for white blood cells. Using special fluorescence staining, peripheral blood micronuclei will be determined by

flow cytometry to estimate benzene genotoxicity. By determining of the shape of the dose response curve for hematotoxicity and genotoxicity for each of the 34 strains we will determine the differences between individual strains and susceptibility to benzene toxicity. Using haplotype association mapping as described in Project 2, we can use quantitative trait analysis to identify potential candidate genes that may be causally related to the benzene toxicity phenotypes under investigation.

Significance and Expected Outcome

Two independent research projects are in progress: (1) oral 14C-benzene ADME (Project 1) and (2) haplotype association mapping (Project 2) in 18 isogenic strains (in progress as described above). This 28-day benzene exposure will provide evidence for low level benzene exposures on hematotoxicity (absolute quantities of peripheral circulating cells by differential cell count and bone marrow hematopoietic lineage) and genotoxicity (micronucleus data by fluorescence cytometry) that simulate human population exposures for haplotype-phenotype association analysis. These data will reveal human orthologs that be further investigated by epidemiology of exposed human populations.

Mouse strains:

1 129S1/SvImJ
2 A/J
3 AKR/J
4 BALB/cByJ
5 C3H/HeJ
6 DBA/2J
7 FVB/NJ
8 NOD.B10/LtJ
9 BTBRT+tf/J
10 KK/HIJ
11 NZW/LacJ
12 CAST/EiJ
13 MOLF/EiJ
14 PWK/PhJ
15 WSB/EiJ
16 C57BL/6
17 NZO/HiLtJ
18 B6C3F1
19 PWK/PhJ
20 CBA/J
21 MRL/J
22 PL/J
23 BUB/BnJ
24 RIIIS/J
25 SJL/J
26 SWR/J
27 NON/LtJ
28 C57BL/10J
29 C57BLKS/J
30 C57BR/cdJ
31 C57L/J
32 LP/J
33 P/J
34 SM/J

PWK/PhJ may replace PWD/PhJ due to introgression

Current Activities and Future Plans

The C57BL/6, C3H/HeJ, and their F1 hybrids are currently being exposed to validate benzene vapor generation, target exposure levels, and practice collection of tissues for analysis prior to initiation of investigation of 34 inbred mouse strain as a model for the human population. Haplotype association mapping analysis will be carried out on those quantitative phenotypes that show significant statistical significant differences between strains. Single nucleotide polymorphisms and copy number variants in the 34 inbred strains phenotypes will be analyzed for their association with identified quantitative measures of genotoxicity, hematotoxicity, and hemoglobin adducts as described. Identification of candidate genes in those sequence intervals showing significant association ($-\log_{10}$ P-value) will be investigated using SNPster. If strong candidates are identified with that demonstrate strong effects (>15-25% size effect for the contribution of genetic variation using linear regression models), bioinformatic analyses will be

carried out to further refine candidates and *in vitro* and *in vivo* studies protocols may be designed and peer reviewed for functional validation.

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